Carbohydrate Research, 83 (1980) 335–349
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

COUNTER-ION DEPENDENCE IN SALT-FREE, AQUEOUS SOLUTIONS OF HEPARIN*

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(Received June 6th, 1979, accepted for publication in revised form, December 15th, 1979)

ABSTRACT

Chiroptical properties of heparin for various degrees of neutralization of the sulfate and carboxylic groups and for different counter-ions in salt-free aqueous solutions were investigated Variations of optical rotation and ellipticity values at given wavelengths are compared to simultaneous pH and viscosity changes observed during the neutralization of heparin by sodium and calcium hydroxide For Na^+, variations of ellipticity at 210 nm are related to acid–base properties of uronic carboxylic groups C d characteristics found for alkaline-earth counter-ions (Mg^{2+}, Ca^{2+}, and Ba^{2+}), as compared to Na^+, are assigned to effects of divalent ions on the ionization behavior of carboxylic groups Among the divalent counter-ions considered, Ca^{2+} gave the strongest interaction with the heparin polyanion, but no specific complex formation was observed O r d and c d data are discussed on the basis of a randomly coiled structure for macromolecules composed of rigid, heterocyclic repeating-units that are independent of each other in so far as electronic transitions of chromophore groups contributing to optical activity are concerned

INTRODUCTION

Because of its outstanding anticoagulant properties, heparin, a polysaccharide extracted from various tissues, is now an industrial compound available in different salt forms (Na^+, Ca^{2+}, *etc*), which are used on a large scale in the medical field Although this highly charged macromolecule, which bears sulfate and carboxylate groups, was discovered early at the beginning of this century, relatively little interest has been devoted to its polyelectrolytic behavior as compared to its biological activity[1]

At present, heparin is considered as a macromolecule predominantly composed of alternating sequences of α-L-idopyranosyluronic acid 2-sulfate and 2-deoxy-2-sulfamino-α-D-glucopyranosyl 6-sulfate residues glycosidically (1→4)-linked Other

*Acid–base and chiroptical properties of heparinic acid, the acid form of heparin Part I
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constituents are present, such as β-D-glucopyranosyluronic acid and 2-acetamido-2-deoxy-α-D-glucopyranosyl residues, depending on the origin of the compound[2] Concerning the polyelectrolytic behavior of heparins, the ionized groups present are OSO_3^-, $NHSO_3^-$, and CO_2^-, in proportions not yet exactly defined Based on optical activity, heparin is a rather complex polymer, and its conformational structure in solution is still under discussion Based on the chiroptical properties of its complexes with dyes, heparin has been suggested to be a helical polymer[3] or, at least, a polymer having a high degree of helical order[4] In contrast, a random (or Gaussian) coil conformation has been proposed on the basis of the physicochemical properties of its solutions[1] Chiroptical data (o r d and c d) have been widely applied to the study of the conformational behavior of poly-α-amino acid- and polynucleotide-type polyelectrolytes[5] On the other hand, it has been shown that valuable information on the chemical properties of optically active polyelectrolytes may be drawn from o.r d and c d data for randomly coiled compounds[6] In spite of its heterogeneous chain-lengths and chemical composition, heparin may be considered as another example of multifunctional, optically active polyelectrolytes Therefore, it was of interest to determine whether pH-induced, chiroptical-property changes in heparin could be related to acid–base properties of the various functional groups, as is the case for synthetic, optically active polyelectrolytes So far, interest in the protonic form of heparin has been limited, probably because of the lability of this compound, from which the sulfate groups are split off very rapidly at low pH[7] This paper reports the chiroptical properties of heparin in salt-free water solutions, at various degrees of neutralization and for different counter-ions, with special interest in the composition of Ca^{2+} and Na^+ systems For the sake of homogeneity, all the experiments reported herein were carried out on a single sample of heparin extracted from porcine-mucosa tissues and supplied as the calcium salt The stability of the acid form of heparin in water solution was first investigated in order to ascertain the experimental conditions allowing the effect of degradation during observation to be neglected

EXPERIMENTAL

Materials — The sample of heparin (Ca^{2+} salt, or calcium heparinate) was kindly supplied by Choay Chimie S A (75782 Paris, France, Calciparine Choay) [*Anal* Found N, 2 26, S, 11 65, biological activity, 158 u s p units/mg (167.6 units/mg for the fully dried compound)]

The ^1H- and ^{13}C-n m r data indicated that the selected heparin belongs to type A according to Perlin[2], *i e* , it contains an important proportion of 2-acetamido-2-deoxy-α-D-glucopyranosyl residues A solution of the calcium salt was prepared by dialyzing, for 48 h, a solution (*c* 1 1) in a semipermeable cellulose tube against a slow flow of distilled water to yield a stock solution (T_N 0 05 equiv L^{-1})

A solution of the acid form of heparin (heparinic acid) was prepared by passing the stock solution of calcium salt through a column containing Dowex 50W X-8 (H^+ form, 20–50 mesh) ion-exchange resin The column was washed and the collected

solution was diluted to 100 mL The solution thus obtained was titrated with 0.1M sodium hydroxide and adjusted to the desired concentration by dilution

Solutions of various salts of heparin (Na^+, Mg^{2+}, and Ba^{2+}) were obtained by passing the stock solution of the calcium salt through columns of Dowex 50W X-8 (20–50 mesh) resin of the appropriate ionic form

The titrated solutions of sodium hydroxide were prepared from standard M solutions by appropriate dilution with carbon dioxide-free distilled water The titrated solutions of calcium hydroxide were obtained from carbon dioxide-free distilled water saturated with calcium oxide

Methods — Potentiometric titrations were performed at 25° with a Radiometer pH M-52 pH-meter, fitted with a Metrohm EA 121 glass-electrode This electrode was standardized at pH 4 005 (50mM potassium hydrogenphthalate buffer) and at pH 9.196 (50mM borax solution) The determinations of pH were performed on solutions maintained under an atmosphere of nitrogen after stirring had been stopped The viscometric measurements were performed at 25° with a FICA 52 000 "Viscomatic" viscometer The o r d curves were recorded with a FICA "Spectropol I" spectropolarimeter at 25° Optical rotations were determined at a fixed wavelength with the same instrument by setting the monochromator at the wavelength selected The c d curves were recorded with a Jasco J-40 B dichrometer at room temperature (22–23°), the double monochromator and cell compartment were purged with nitrogen The effect of temperature on ellipticity at 210 nm was measured with a double-wall cell, connected to a thermostat ^{1}H- and ^{13}C-n m r spectra were recorded with a Brucker WH-90 spectrometer operated by the Service Commun "Recherches" de l'Université de Rouen

RESULTS AND DISCUSSION

Heparinic acid is not stable in salt-free water solutions its biological activity decreases rapidly with time[1 8] A release of sulfuric acid was shown[7], which resulted

TABLE I

VARIATION OF THE BIOLOGICAL ACTIVITY OF HEPARINIC ACID[a] WITH TIME

Time (h)	*Biological activity (u s p units/mg)*
0	158
4	156
24	143
72	130
168	116
336	76

[a] T_N 26 mequiv L^{-1}

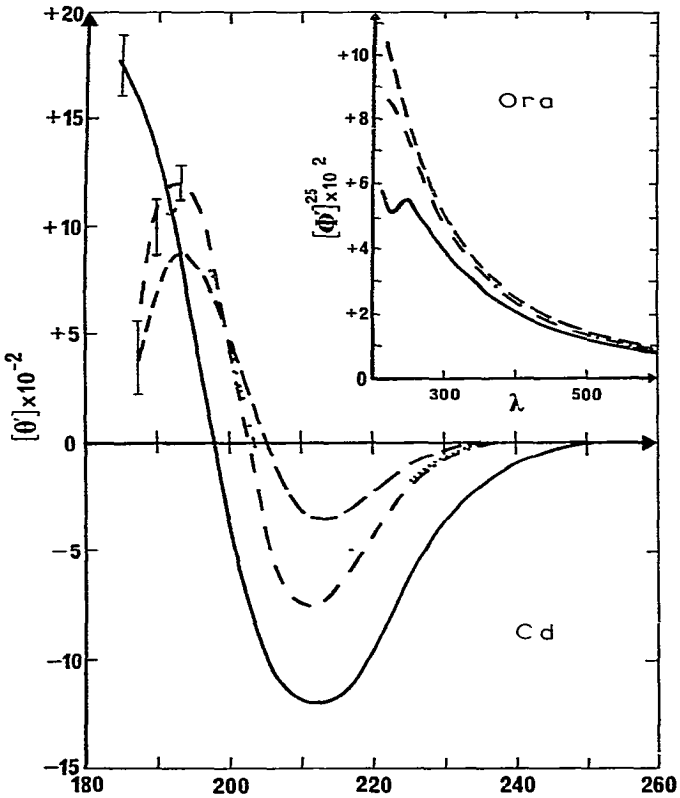

Fig 1 O r d and c d spectra of heparinic acid (———), sodium heparinate (– – –), magnesium heparinate ( ), and calcium heparinate (– – – –) in salt-free aqueous solution (T_N 26 mequiv L^{-1})

primarily from the H^+-catalyzed hydrolysis of *N*-sulfate groups In order to evaluate the degradation under the conditions required for chiroptical measurement (2 h and T_N 10–1mN), the decrease of activity *vs* time was monitored by biological tests A solution (26mN) of heparinic acid in water was kept for 14 days at room temperature Samples were withdrawn from the medium at various intervals, and the acid neutralized to pH 7 with sodium hydroxide The resulting heparin solutions were tested to evaluate the remaining biological activity (see Table I) The biological activity at a selected concentration may be considered as constant for measurements performed within 2 h after the preparation of the solution This condition was observed for all experiments reported herein

The o r d and c d curves for heparinic acid, and the sodium, calcium, and magnesium salts (c d only) are shown in Fig 1 Usually o r d and c d data of polymers are referred to as repeat units and expressed in terms of monomolar optical rotation $[\phi] = \alpha_{exp} \times$ m r w $/l\,c$, and of monomolar ellipticity $[\theta] = \theta \times$ m r w $/l\,c$, with m r w = mean residue mol wt , l = path-length in dm, and c = conc in g/100 mL For heparin or heparinic acid, where the nature of the various repeating-units is not well identified, it was difficult to ascribe a value to m r.w The values used so

far have been scattered 563 (ref 9), 537 (ref 10), and 1170 ±50/2 (ref. 11) On the other hand, heparinates are hygroscopic compounds that often contain salt and ethanol as impurities In order to avoid the uncertainty in the concentrations of heparinate solutions, the o r d and c d data reported in the present study have been referred to the normality (T_N) of the solution, as deduced from titration curves of corresponding solutions of heparinic acid The data have been expressed* by $[\phi'] = 10 \times \alpha/l\, T_N$ and $[\theta'] = 10 \times \theta/l\, T_N$

The o r d curves of heparinic acid and its sodium and calcium salts are positive For the calcium salt, the curve is plain over the spectral range considered, whereas a maximum at 250 nm and a shoulder in the same zone are observed for the acid and the sodium salt, respectively For all three compounds, the optical rotations are almost identical in the visible part of the spectrum ($\lambda > 360$ nm), whereas they differ widely in the u v range

The c d spectra of heparinic acid, and of the sodium, calcium, and magnesium salts show two bands, of opposite signs The first band, located at 210 nm, is negative and its magnitude depends on the counter-ion The second band is located at lower nm-values The corresponding maximum, located at 195 nm, depends on the counter-ion For heparinic acid, the maximum of the second c d band, below 185 nm, was not reached These results agree with data reported in the literature for heparinic acid and the sodium salt[4 12]

Unsubstituted polysaccharides do not show any c d band in the 185–250-nm region of the spectrum[13] Therefore, the bands reported in Fig 1 result from the interaction of circularly polarized electromagnetic waves with chromophores of the substituent groups bound to the polysaccharide backbone These c d bands were found to be slightly temperature-dependent In the range of 20–60°, the changes were linear and independent of the counter-ion (see Fig 2) This observation indicates that no order-to-disorder, conformational transition occurred in the range of temperature considered The great similarity of the slopes $\Delta[\theta']/\Delta t$ for the three salts is evidence that the structures of these compounds are fundamentally identical In contrast, the slope is different for heparinic acid, probably reflecting the lack of ionization of the carboxylic groups Heparin c d bands have been described as "amide-Cotton effects"[12] and "amide-like Cotton effects"[14] Actually, several chromophores present within the heparin or heparinic acid molecule may be asymmetrically perturbed by the different chiral centers of pyranosidic rings It is well known that optically active carboxylic acid, carboxylate, and amide groups of low-molecular-weight compounds show c d bands at >185 nm So far, the sulfamido chromophore has been little investigated, but it is likely that it also contributes to the absorption of heparin compounds in the accessible part of the spectrum[14] Therefore, the c d spectra shown in Fig 1 do not correspond to an exciton-split

*Classical, monomolar optical rotation $[\phi]$ and monomolar ellipticity $[\theta]$ are given by the relations $[\phi] = [\phi'] \times \overline{d\,s}$ and $[\theta] = [\theta'] \times \overline{d\,s}$, respectively, where $\overline{d\,s}$ is the accurately determined, mean-degree of substitution of the pyranose ring by acidic groups

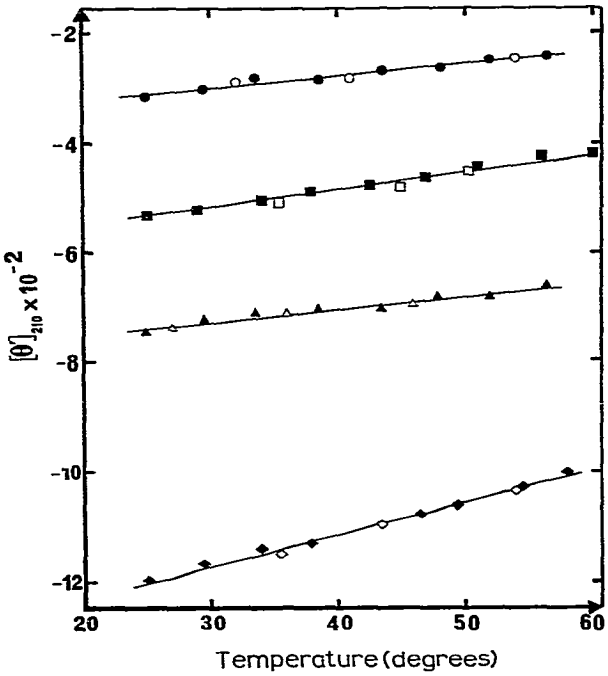

Fig 2 Variation of the equivalent-gram ellipticity at 210 nm ($[\theta']_{210}$) as a function of temperature for heparinic acid and some of its salts (T_N 5 mequiv L^{-1}), (◆ ◇), heparinic acid, (▲ △), sodium salt, (■ □), magnesium salt, and (● ○), calcium salt (full symbols correspond to increasing, open symbols to decreasing temperature)

electronic transition or to electronic transitions of one chromophore only The bands certainly result from the contributions of several chromophores, including the carboxylic or carboxylate groups of the L-iduronic and D-glucuronic residues, and of the amide groups that are present in the 2-acetamido-2-deoxy-D-glucose residues of the sample calcium heparinate investigated, as shown by n m r spectrometry Similar, overlapping electronic-transition states have been observed for synthetic polyacids; however, the pH-induced c d changes could be readily related to the ionization of different acidic groups present in the macromolecules[6 15]

Basically, both conformational and chemical factors may contribute to pH-induced changes of optical activity However, in the absence of any ordered conformation that perturbs chromophores specifically, it was shown that repeating-units of an optically active polymer contribute almost independently to the total optical activity A simple method for distinguishing the respective contributions of macromolecular conformations and chemical modifications consists in comparing the chiroptical-property changes with the ionization states of the acidic groups, these may be optically active chromophores themselves or may indirectly perturb optically active chromophores[6] Thus, the changes in the chiroptical properties of heparinic acid in solution during the neutralization of sulfate and carboxyl groups were studied As our aim was to correlate chiroptical-property changes with polyelectrolytical behavior,

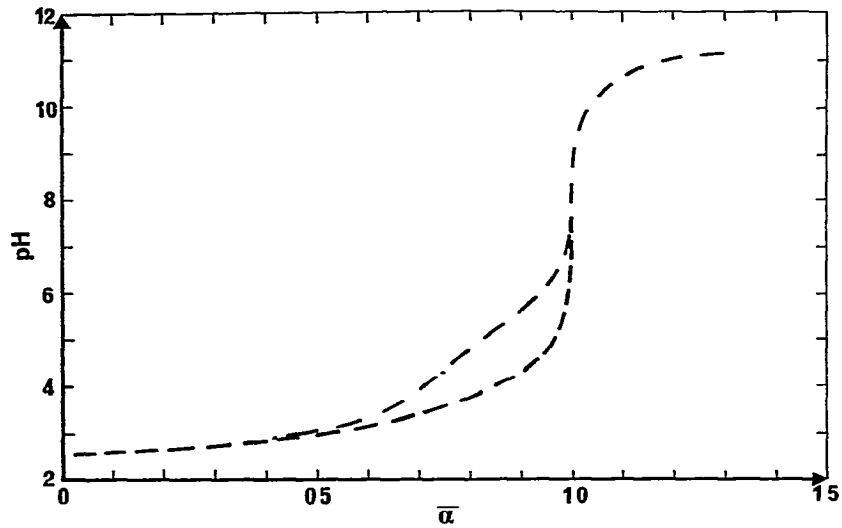

Fig 3 Potentiometric titration curves of heparinic acid (T_N 5 2 mequiv L^{-1}) neutralized by sodium hydroxide (– – –) and calcium hydroxide (———) at 25° in salt-free water solution, $\bar{\alpha}$ = number of added equiv of base per number of equiv of acid to be neutralized The degree of neutralization was adjusted by adding dropwise solutions of free bases (41 8mM)

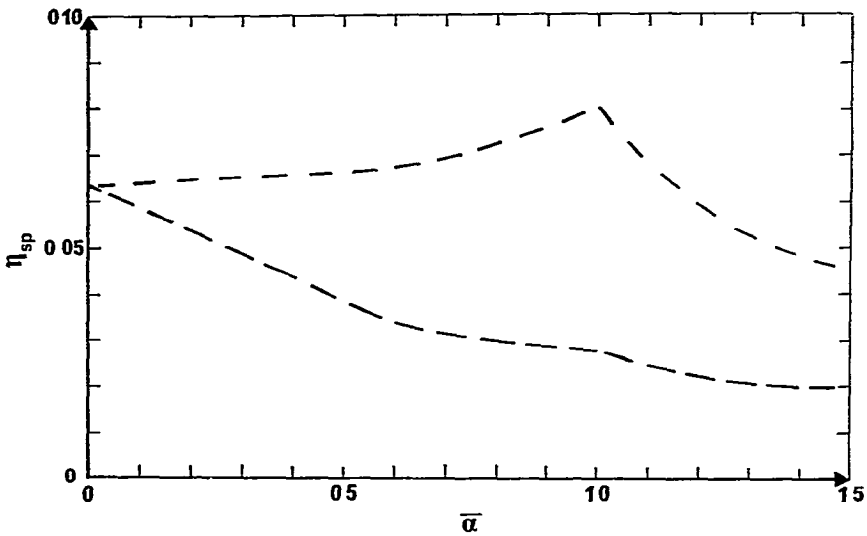

Fig 4 Viscometric titration curves of heparinic acid (T_N 5 2 mequiv L^{-1}) neutralized by sodium hydroxide (– – –) and calcium hydroxide (———) at 25° in salt-free water solution

the neutralization by sodium and calcium hydroxide was monitored not only by polarimetry and dichrometry, but also by viscometry and potentiometry, the concentration (T_N 5 2 mequiv.L^{-1}) being the same for all experiments

In the potentiometric titration of heparinic acid (see Fig 3) as a function of neutralization, no difference was detected between the Na^+ and Ca^{2+} curves, in the

range $0 < \bar{\alpha} < 0.4$ The two curves became separate at $\bar{\alpha} \sim 0.4$, the separation increasing and then decreasing as α increased, and the curves merging again at $\bar{\alpha}$ 1 Although no clear potential jump was observed, it is obvious that the S-shaped part of the Na^+ curve (in the range $0.6 < \bar{\alpha} < 0.8$) corresponds to the beginning of the ionization of the carboxyl groups, as previously reported[7] The lowering of the pH values, which flattened the Ca^{2+} titration curve for $\bar{\alpha} > 0.4$, agrees well with the usual effect of alkaline-earth cations on potentiometric titration curves of polycarboxylic acids[16,17] On the other hand, the lack of dependence of the titration curve of heparinic acid on the counter-ion at low $\bar{\alpha}$ agrees well with the neutralization of permanently ionized sulfate groups Indeed, these strongly acidic groups are known to be only slightly sensitive to the nature of counter-ions in polysulfates[16]

The viscometric titration curves for heparinic acid with sodium and calcium hydroxides are shown in Fig 4 For Na^+, the specific viscosity (η_{sp}) rose slightly as $\bar{\alpha}$ increased from 0 to 0.6, *i e*, when the strongly acidic groups were neutralized In that part of the curve, the charge density remained constant At $\bar{\alpha} \sim 0.6$, the carboxylic groups started to be ionized In a salt-free system, the new electric charges increase the electrostatic repulsive forces and, thus, produce a further extension of the already partially extended macromolecules Beyond $\bar{\alpha}$ 1, the specific viscosity decreased because of the salt-effect of the excess of alkaline reagent, which screened electric charges These observations agree well with the polyelectrolytical behavior to be expected from such a multifunctional compound as heparinic acid The Ca^{2+} viscometric curve was completely different from that for Na^+ Although a change of slope still occurred at $\bar{\alpha}$ 0.6, the specific viscosity decreased immediately as $\bar{\alpha}$ increased from 0 Furthermore, only a weak break was observed at $\bar{\alpha}$ 1, which marked the end of the neutralization of the acidic groups and the beginning of the salt-effect of the

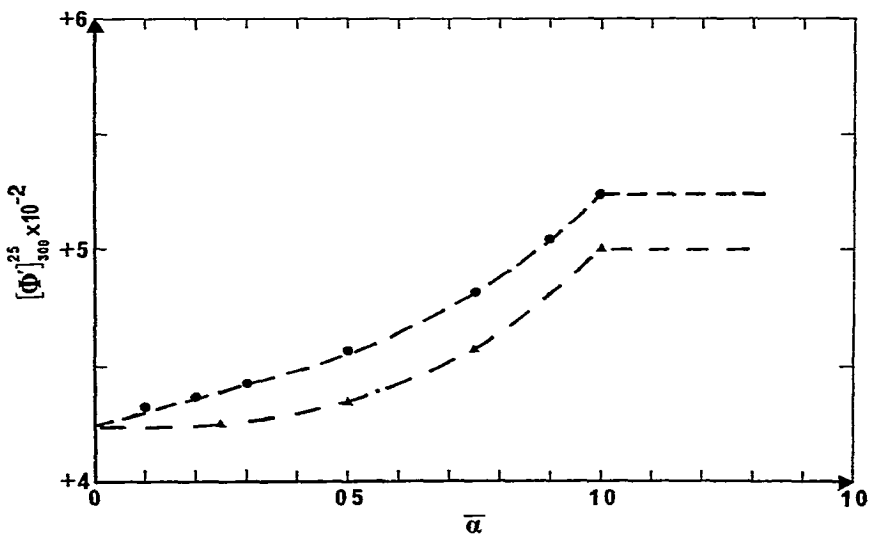

Fig 5 Polarimetric titration curves of heparinic acid (T_N 5.2 mequiv L^{-1}) neutralized by sodium hydroxide (– – –) and calcium hydroxide (————) at 25° in salt-free water solution

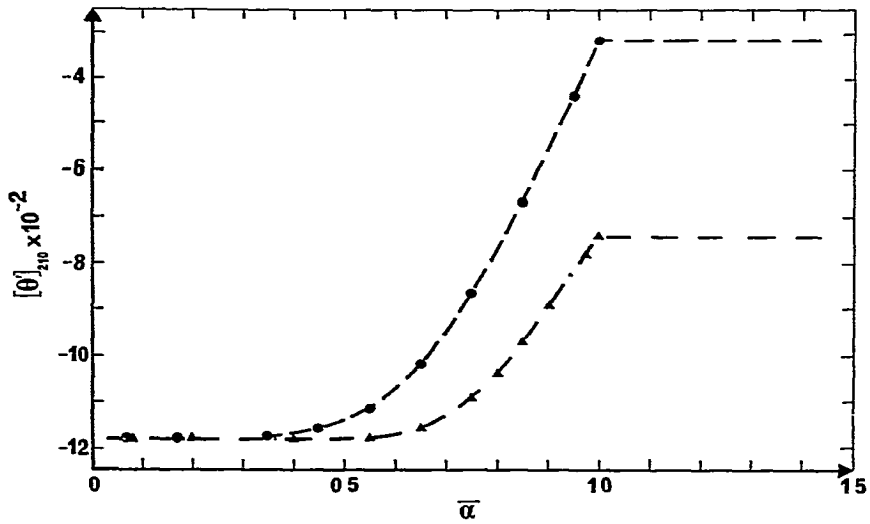

Fig 6 Dichrometric titration curves of heparinic acid (T_N 5 2 mequiv L^{-1}) neutralized by sodium hydroxide (– – –) and calcium hydroxide (–––) at room temperature in salt-free water solutions

excess of calcium hydroxide Whatever the value of $\bar{\alpha}$, the specific viscosity was lower for Ca^{2+} than for Na^{+}, the difference being greatest at $\bar{\alpha}$ 1 This trend, generally observed for polyanion–Ca^{2+} systems[18], indicates a strong interaction (but not necessarily a complex formation[19]) that allows macromolecules to contract[20]

The polarimetric titration of heparinic acid (see Fig 5) was performed either by adding the alkaline reagent to the polyacid solution or by mixing, in appropriate proportions, solutions of heparinic acid and of its salts ($0 < \bar{\alpha} < 1$) Both methods gave the same results For Na^{+}, a smooth enhancement of the optical rotation was observed with increase of $\bar{\alpha}$ up to a value of 1 The trend was accentuated for Ca^{2+} Beyond $\bar{\alpha}$ 1, the optical rotation remained constant whatever the degree of neutralization, while the specific viscosity decreased in the same zone, *i e*, the conformations of the poly-ion continued to change

In the dichrometric titration of heparinic acid with sodium and calcium hydroxide, the two curves (see Fig 6) are similar to the corresponding curves obtained by polarimetry, but slight differences may be emphasized for Na^{+}, the ellipticity remained constant for $0 < \bar{\alpha} < 0\,6$, whereas for Ca^{2+} it increased as early as $\bar{\alpha} \sim 0\,4$ For both cations, no variations were found for $\bar{\alpha} > 1$, thus confirming that no relation between conformation and optical activity exists for $\bar{\alpha}$ values higher than 1 In these comparative experiments, the ionization of carboxylic acid groups, which occurs in the range $0\,6 < \bar{\alpha} < 1$, appears to be a critical factor for both viscometric titration curves, and for potentiometric and dichrometric curves obtained by sodium hydroxide titration In contrast, potentiometric and dichrometric curves corresponding to Ca^{2+} did not show any particular features for $\bar{\alpha} \sim 0\,6$–$0\,7$, and diverged from the Na^{+} curves at similar $\bar{\alpha}$ values ($\bar{\alpha} \sim 0\,4$). The unusual variation of c d and o r.d properties of sodium heparinate with pH led Stone[4,12] to suggest a conformational transition,

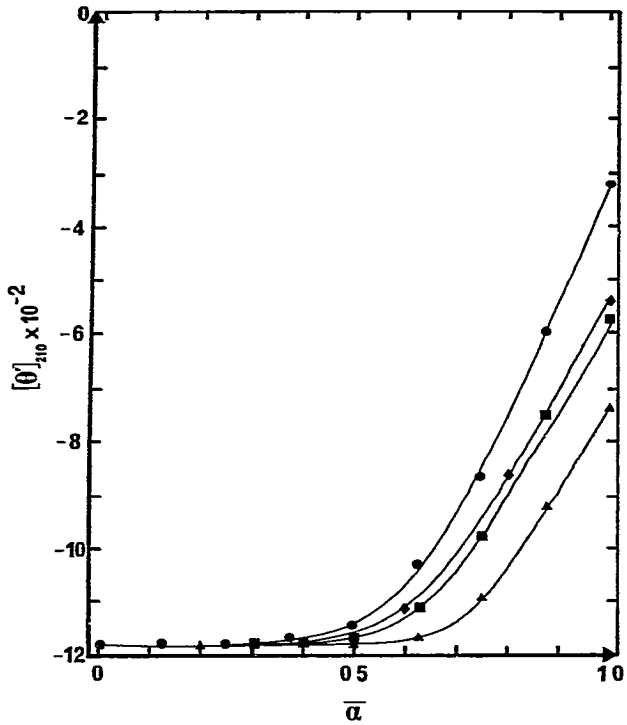

Fig 7 Variation of $[\theta']_{210}$ as a function of the degree of neutralization (mixing method) in salt-free water solution (T_N 2 5 mequiv L^{-1}) for various counter-ions (▲), Na^+, (■), Mg^{2+}, (◆), Ba^{2+}, and (●), Ca^{2+}

on the assumption that the 210-nm Cotton effect was largely due to an amide transition The differences between the Ca^{2+} and Na^+ titration curves just described do not show a particular, order-to-disorder conformational transition Furthermore, these differences are not specific for Ca^{2+} ions, since they depend on the cation in the alkaline-earth series. Dichrometric titration of heparinic acid was performed by the mixing method for Mg^{2+}, Ba^{2+}, and Ca^{2+}, as compared to Na^+ (see Fig 7), the concentration of heparinic acid being half that corresponding to the experiment described in Fig 6 No significant effect of the concentration was detected for the Na^+ and Ca^{2+} systems The Mg^{2+} and Ba^{2+} curves were similar, located between the curves for Na^+ and Ca^{2+}, and the four curves presented similar features At low $\bar{\alpha}$ values, the ellipticity at 210 nm was independent of the degree of neutralization and of the counter-ion At high $\bar{\alpha}$ values, the ellipticity was enhanced progressively up to the specific values of the corresponding heparinic acid salts ($\bar{\alpha}$ 1) In the latter part of the curves, the dichrometric titration of heparinic acid was cation-dependent Furthermore, the $\bar{\alpha}$ values at which the curves for Mg^{2+}, Ba^{2+}, and Ca^{2+} diverged from that for Na^+ increased according to the series $Ca^{2+} < Ba^{2+} < Mg^{2+}$

Park and Chakrabarti[9] have suggested that pH-induced variations of the 210-nm c d. band of sodium heparinate are directly related to the ionization behavior

of the carboxylic chromophores Such an interpretation rules out the possibility that an order-to-disorder, conformational transition is the source of the c d changes observed for the Na^+ salt of heparinic acid In that sense, sodium heparinate behaves like all optically active polyelectrolytes studied by us[6,15,21] Thus, the lack of dependence of ellipticity on the degree of neutralization and on the counter-ion at low $\bar{\alpha}$ values agrees well with the neutralization of sulfate groups only, as these groups remain ionized whatever the counter-ion, including H^+ The changes of ellipticity observed for alkaline-earth counter-ions in the range of intermediate values ($0\,4 < \bar{\alpha} < 0\,6$), *i e*, in a range where only sulfate groups are neutralized in the case of Na^+, could be assigned to chromophoric modifications due to specific interaction (or complex formation between macromolecules and divalent cations) However, these changes may result from the chemical modification of carboxylic group chromophores only, as in the case of Na^+ Indeed, the acidity of carboxylic groups of polycarboxylic acids is drastically enhanced in the presence of such divalent counterions as Ca^{2+} or Ba^{2+} as compared to monovalent ones The effect of Ca^{2+} on the strength of the carboxylic-group acidity of uronic acid residues present in heparinic acid molecules is clearly shown in Fig 3. Therefore, in the presence of alkaline-earth counterions, the uronic acid groups are allowed to ionize at lower pH values and, thus, they compete with sulfate groups in the neutralization of the added divalent bases

In the absence of any cooperative conformational transition, the optical activity, at a given wavelength, of a partially ionized polyelectrolyte results, in principle, from a simple summation of the contribution from ionized and non-ionized repeating-units, according to their respective weights Some discrepancies may be observed in the presence of a neighboring-group effect of vicinal residues that modify the specific contribution of a given residue[22] For heparinic acid, a simple additivity is to be expected because the uronic acid residues are rather far from each other Assuming that $[\theta_0']_{210}$ (the equiv gram ellipticity of heparinic acid at $\bar{\alpha}$ 0, and at 210 nm) and $[\theta_s']_{210}$ (the equiv gram ellipticity of salts at $\bar{\alpha}$ 1) are characteristic of contributions of non-ionized and ionized uronic acid residues, respectively, the degree of ionization (α_{CO_2H}) of corresponding carboxylic groups is given by Eq *1*,

$$\alpha_{CO_2H} = ([\theta']_{210} - [\theta_0']_{210})/([\theta_s']_{210} - [\theta_0']_{210}) \qquad (1)$$

where $[\theta']_{210}$ is the equiv gram ellipticity at a given $\bar{\alpha}$ value Fig 8 shows the degrees of ionization of carboxylic groups as a function of the degree of neutralization $\bar{\alpha}$ of heparinic acid for different counterions, as deduced from the data of Fig 7 by use of Eq *1* For Na^+, the variation was linear in the range $0\,75 < \bar{\alpha} < 1$, as would be expected for the neutralization of a weakly acidic carboxylic group located in a highly negatively-charged polyelectrolyte, at pH values where the self-ionization of the carboxylic group may be neglected The deviation from linearity observed below $\bar{\alpha} \sim 0\,75$ may be ascribed to the self-ionization of carboxylic groups that becomes far from negligible as the pH decreases The extrapolation of the linear part of the curve showed that, in the absence of self-ionization, α_{CO_2H} would be 0 at $\bar{\alpha}$ 0 7, a value that is in good agreement with the inflexion in the potentiometric titration

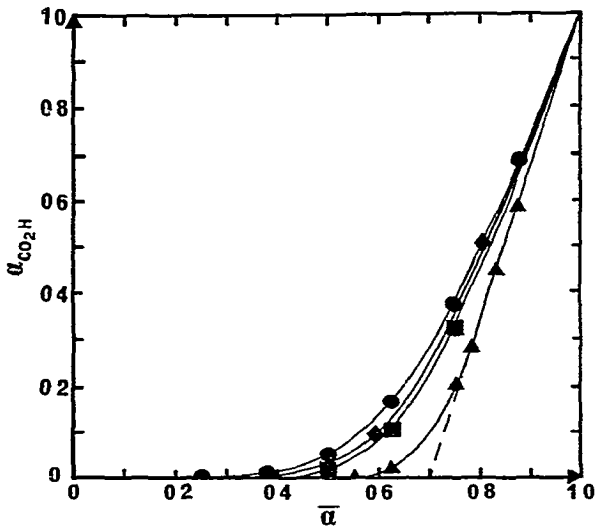

Fig 8 Values of the degree of ionization of the carboxylic groups of heparinic acid α_{CO_2H} (deduced from data in Fig 6 by use of Eq *1*) as a function of the degree of neutralization of heparinic acid by various counter-ions (▲), Na^+, (■), Mg^{2+}, (◆), Ba^{2+}, and (●), Ca^{2+}

curve (see Fig 3) Furthermore, this extrapolated value corresponds to a ratio of CO_2H SO_3^- of between 1 2 and 1 3, which agrees with the complex structure proposed for heparin[23]

The curves corresponding to alkaline-earth counterions merged tangentially with the linear part of the Na^+ curve for $\bar{\alpha}$ 1 The larger deviations from linearity observed for divalent cations, as compared to Na^+, would result from a greater ionization of the carboxylic groups for a given value of the degree of neutralization This interpretation is supported by the shift, as compared to Na^+, of the potentiometric curve for Ca^{2+} counter-ions to lower pH values in the range 0 6 $< \bar{\alpha} <$ 1 (Fig 3) This interpretation, however, does not account for the cation-dependence of $[\theta'_s]$, which was taken as corresponding to 100% of ionized groups for any cation The existence of specific interactions (complex formation) between macromolecules and counter-ions was excluded Indeed, only the magnitude of the c d bands was affected, while the positions of the maxima remained unchanged (Fig 1) For Na^+ and Ca^{2+} polyuronates, the differences in c d values were assigned to intermacromolecular aggregation *via* Ca^{2+} ions, as shown by the ultracentrifugation of calcium–polyuronic acid systems[24] The ultracentrifugation of calcium heparinate solutions carried out under similar conditions did not show any intermacromolecular interactions. On the other hand, it has been shown that the Na^+ and Ca^{2+} activity coefficients (γ) for sodium heparinate[25,26] (0 40) and calcium heparinate[26] (0 17) agree with those expected for a highly charged polyelectrolyte, taking into account the electrostatic effects[17] On this basis, it is suggested that the cation-dependence of the c d spectrum of heparin results from the presence of counter-ions closely surrounding the polyion, and particularly of carboxylic groups, as previously sug-

gested[1,27] Indeed, the chiroptical characteristics of chromophore groups depend not only on conformation, but also on properties of the medium (dielectric constant, polarizability, *etc*), especially where these chromophores, such as the carboxylic groups of heparin, are not included within a rigid system but one merely bonded to it

Two observations are in favor of this interpretation Firstly, the significant effect of Ca^{2+}, as compared to Na^{+}, on solvent–macromolecule interactions, this effect has previously been emphasized, on the basis of light-scattering experiments[28] Secondly, the agreement between the order $Na^{+} < Mg^{2+} < Ba^{2+} < Ca^{2+}$ [which is deduced either from the value of $[\theta'_s]_{210}$ for $\bar{\alpha}$ 1 (Fig 7), or from the deviation from linearity, or from the $\bar{\alpha}$ value where α_{CO_2H} becomes nil (Fig 8)], and that obtained from the affinity of heparin for various counter-ions as determined by ion-exchange[29] or by ^{23}Na-n m r spectrometry[30] In spite of this agreement, the following observations concerning optical activity are relevant

The o r d. spectra of heparinic acid and its sodium and calcium salts were almost identical for $\lambda > 400$ nm, whereas they were increasingly different as λ decreased below this value Quantitative comparison of o r d and c d spectra through Kronig–Kramers transforms was not possible because of the complexity of the c d bands, which result from overlapping electronic transitions (as previously stated) Qualitative comparison showed that the o r d spectra for $\lambda < 360$ nm reflected the pH- and counter-ion-dependence of c d bands observed in the u v region in the 185–230-nm spectral range However, the correlation between o r d changes and variations of the degree of ionization of uronic carboxylic groups was not complete Indeed, the optical rotations began to change at 300 nm while the degree of ionization α_{CO_2H} was still 0 (Fig 5) To account for the optical activity of heparin $\lambda > 360$ nm, it is suggested that the o r d spectrum depends on strong Cotton effects located at <185 nm, the contribution of partial o r d due to the 200-nm c d bands becoming negligible obviously because of the dispersive effect The fact that the strong Cotton effects in the far-u v range are almost independent of the pH and of the counter-ion could be considered to favor a rigid macromolecular conformation Such a structure would rule out the interpretation that pH-induced chiroptical-property changes in heparinic acid are predominantly based on chemical factors However, the heparin molecule is composed of pyranose rings linked through glycoside bonds Therefore, the magnitudes of the strong Cotton effects in the far-u v range may be controlled simply by the conformations and configurations of the various rigid rings, which are independent of each other in so far as optical activity is concerned, because of the mobility of glycoside bonds Induced circular dichroism (i c d), observed for dyes interacting with heparin, has been considered to result from a degree of helical order in the macromolecules[4] It must be pointed out, however, that i c d bands were also observed for basic dyes in the presence of disordered, optically active systems, such as L-sodium tartrate[31], micellar solutions of chiral surfactant[32], and optically active, synthetic polyanions[33] Therefore, the presence of i.c.d. bands would not be in disagreement with a model based on flexible, optically active macromolecules compared of rigid rings.

The observation that the respective contributions of the ionization of D-glucuronic and L-iduronic carboxylic groups (known to be present in heparin extracted from porcine mucosal tissues) were not distinguished in dichrometric titration curves may be discussed on the basis of the considerations above It is likely that the carboxylic groups in both types of uronic unit behave similarly, in so far as the ionization process is concerned Indeed, that process depends primarily on the high negative charge of the heparin polyanion Both groups are certainly ionized in the same range of pH values Accordingly, the observed c d changes represent averages that reflect the total ionization of carboxylic groups

In conclusion, the present interpretation accounts well for the results of the chiroptical investigations described in the present paper The conformation proposed for heparin in solution agrees with the Gaussian coil suggested from partial specific volume, sedimentation coefficient, molecular weight, and intrinsic viscosity measurements[1-8]. It also agrees with recently reported n m r. investigations[2-7] on the polyanionic character of heparin

ACKNOWLEDGMENTS

The authors are indebted to Dr J Choay and to Choay S A for the supply of samples of calcium heparinate, for performing biological tests, and for the financial support of this work

REFERENCES

1 J EHRLICH AND S S STIVALA, *J Pharm Sci*, 62 (1973) 517–544
2 A S PERLIN, *Fed Proc Am Soc Exp Biol*, 36 (1977) 106–109
3 S HIRANO AND K ONODERA, *Life Sci*, 6 (1967) 2177–2183
4 A L STONE, *Fed Proc Am Soc Exp Biol*, 36 (1977) 101–106
5 R W WOODY, *J Polym Sci, Macromol Rev*, 12 (1977) 181–320
6 M VERT, in E SELEGNY (Ed), *Polyelectrolytes*, Reidel, Dordrecht, Holland, 1974, pp 347–370
7 J R HELBERT AND M A MARINI, *Biochemistry*, 2 (1963) 1101–1106
8 S S STIVALA, L YUANG, J EHRLICH, AND P A LIBERTI, *Arch Biochem Biophys*, 122 (1967) 32–39
9 J W PARK AND B CHAKRABARTI, *Biochem Biophys Res Commun*, 78 (1977) 604–608
10 M CHING MING CHUNG AND N F ELLERTON, *Biopolymers*, 15 (1976) 1409–1423
11 L YUANG AND S S STIVALA, in R A BRADSHAW AND S WESSLER (Eds), *Heparin Structure, Function and Clinical Implications*, Plenum Press, New York, 1975, pp 39–49
12 A L STONE, *Nature (London)*, 216 (1967) 551–553
13 D BALASUBRAMANIAN AND C KUMAR, *Appl Spectrosc Rev*, 11 (1976) 223–286
14 A L STONE, *Biopolymers*, 10 (1971) 739–751
15 C VILLIERS, C BRAUD, M VERT, AND E CHIELLINI, *Macromolecules*, 12 (1979) 103–107
16 M RINAUDO, in E SELEGNY (Ed), *Polyelectrolytes*, Reidel, Dordrecht, Holland, 1974, pp 157–193
17 M RINAUDO AND M MILAS, in A REMBAUM AND E SELEGNY (Eds), *Polyelectrolytes and Their Applications*, Reidel, Dordrecht, Holland, 1975, pp 31–49
18 C BRAUD, *Eur Polym J*, 11 (1975) 421–427
19 M MANDEL, *J Polym Sci, Part C*, 16 (1967) 2955–2962
20 Z WOJTZAK, *Rocz Chem*, 42 (1968) 1319–1326
21 M VERT AND E SELEGNY, *Eur Polym J*, 7 (1971) 1321–1334
22 C BRAUD AND M. VERT, *Polymer*, 16 (1975) 115–119

23 J KISS, in V V KAKKAR AND D P THOMAS (Eds), *Heparin – Chemistry and Clinical Usage*, Academic Press, London, 1976, pp 3–20
24 R KOHN, *Pure Appl Chem*, 42 (1975) 371–397
25 F ASCOLI, C BOTRE, AND A M LIQUORI, *J Phys Chem*, 65 (1961) 1991–1992
26 Unpublished data
27 A S PERLIN, G K HAMER, B CASU, AND G GATTI, *Am Chem Soc Polym Prep*, 19 (1978) 242–245
28 S P LOUCAS, K C YEH, AND H M HADDAD, *J Pharm Sci*, 60 (1971) 1109–1110
29 J R DUNSTONE, *Biochem J*, 85 (1962) 336–351
30 L HERWATS, P LASZLO, AND P GENARD, *Nouv J Chim*, 1 (1977) 173–176
31 S F MASON, *J Soc Dyers Colour*, 84 (1968) 604–612
32 T TACHIBANA AND K KURIHARA, *Naturwissenschaften*, 63 (1976) 532–533
33 J BEAUMAIS, Dr-es-Sci Thesis, Rouen, France, 1978